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Plastid Development in *Pisum sativum* Leaves during Greening[1]

I. A COMPARISON OF PLASTID POLYPEPTIDE COMPOSITION AND *IN ORGANELLO* TRANSLATION CHARACTERISTICS

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ABSTRACT

Changes in plastid polypeptide composition during greening of etiolated peas were investigated by two-dimensional gel electrophoresis. One hundred of the more than 250 polypeptides which could be detected upon silver staining were followed during plastid development. Thirty-nine polypeptides decreased in abundance on a per organelle basis. Twenty-three of the 46 polypeptides which increased in abundance upon greening could be identified as proteins of the thylakoid membrane. The changes in proteins observed during greening of etiolated leaves corresponded largely to those observed during normal leaf expansion. The origin of some of the polypeptides was traced back by comparing the two-dimensional gels of plastid proteins with *in organello* translation products and with polypeptides which had been synthesized *in vitro* from poly(A$^+$) mRNA preparations and posttranslationally imported by chloroplasts. Some polypeptides were specifically identified in two-dimensional gels by Western blot analysis.

Development of proplastids into photosynthetically active chloroplasts is arrested at an intermediate stage when plants are kept in the dark. Upon illumination these etioplasts differentiate into chloroplasts. Many aspects of this transition have been investigated (for reviews see: 14, 17). Most, but not all of the proteins of the photosynthetic apparatus can be detected in etiolated tissue and isolated etioplasts (8).

However, a simultaneous analysis of the changes occurring in the whole set of polypeptides upon illumination of dark-grown plants is missing. Fractionation of plastid proteins by two-dimensional gel electrophoresis offers a comprehensive method with high resolution (13). Polypeptides and the changes occurring in their abundance can be further characterized by identifying (a) the components of the membrane fraction of chloroplasts, (b) the polypeptides which are synthesized *in organello*, and (c) employing Western blot analysis with specific antibodies. We have used these approaches to characterize the changes in plastid polypeptide composition which occur when dark-grown plants are transferred to the light or when leaves expand in the light.

MATERIALS AND METHODS

Material. Pea plants (*Pisum sativum*, progress No. 9) were grown in vermiculite in growth chambers; light was cycled between 16 h light and 8 h darkness; the temperature was 22°C in the light and 18°C in the dark. The plants grown in continuous darkness were kept at 25°C. Plants were transferred to the growth chamber for illumination as indicated in the figures. On the 11th d after sowing, the buds of the etiolated shoots or the leaves were harvested and used either for the isolation of plastids or for *in organello* translation.

Biochemicals were obtained from Sigma and LKB (ampholines); [^{35}S]-methionine was purchased from New England Nuclear.

Methods: Plastid Isolation. About 15 g of leaf tissue of greened plants or 35 g of etiolated buds were chopped with frequently changed razor blades and homogenized in 25 mM Mes (pH 6.5) (KOH), 10 mM KCl, 320 mM sorbitol, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM $MnCl_2$, 5 mM isoascorbic acid by one pulse of 5 s with a Polytron either on speed 9 (etiolated tissue) or speed 7. The homogenized material was filtered through 6 layers of cheesecloth, 2 layers of Miracloth, and 1 layer of nylon netting. The 2,000*g* pellet of the first centrifugation was resuspended in buffer (as above), layered on top of a 25 to 85% (v/v) Percoll gradient and spun for 10 min at 10,000*g*. Intact plastids were recovered as the lower band and washed twice with uptake buffer (320 mM sorbitol, 50 mM Hepes [pH 8] [KOH]).

Intactness of the chloroplasts isolated from fully greened plants was determined by measuring ferricyanide-dependent oxygen evolution. The rate of oxygen evolution of an aliquot of the chloroplast suspension was compared with that of an aliquot which had been broken by hypoosmotic treatment (7). The percentage of intact chloroplasts was routinely found to be between 85 and 93%. Chl was measured according to Arnon (1) and protein with the BCA-protein-assay-reagent (Pierce Chemical Co.). For determination of organelle number per volume, aliquots of the plastid suspensions were diluted with 320 mM sorbitol, 50 mM Hepes (pH 8) (KOH). The plastids were counted with a counting chamber under the microscope. Other aliquots were used for Chl and protein measurements.

***In Organello* Translation by Isolated Plastids.** *In organello* translation was by a method similar to that described by Mullet *et al.* (11). Plastids equivalent to 100 μg protein were incubated for 15 min at 26°C in the light. The incubation mixtures (250 μl volume) contained 320 mM sorbitol, 50 mM Hepes (pH 8) (KOH), 0.9 mM DTT, 0.3 mM sodium phosphate, 0.2 mM of each amino acid (without methionine), 9.5 mM ATP, 12.5 mM $MgCl_2$, and 50 μCi methionine. After 15 min, 25 μl of 100 mM methionine were added, and the chase period terminated by spinning the intact plastids through a 40% Percoll cushion, followed by washing in uptake buffer and resuspending in a small volume of 5 mM EDTA (pH 8). Aliquots were removed to measure TCA precipitatable incorporation of [^{35}S]methionine or to analyze the translation products by SDS-gel electrophoresis.

Measurement of [^{35}S]Methionine Incorporation. Aliquots of

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the plastids after *in organello*-translation were spotted onto 1 cm^2-squares of 3MM-Whatman paper and dried at room temperature. The filters were then placed into a large volume of 10% TCA and boiled for 10 min, cooled on ice, rinsed with water, and extracted two times in an equal volume of ethyl ether and ethanol, rinsed in ethyl ether, and dried. They were placed in scintillation vials with the appropriate volume of scintillation fluid and counted. Blanks were included throughout the procedure.

SDS Gel Electrophoresis and Autoradiography. Protein samples were solubilized by boiling for 5 min in 2.3% (w/v) SDS, 0.125 M Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 10% (v/v) glycerol. The separating gel contained 12.5% (w/v) acrylamide, 0.33% bisacrylamide, 0.375 M Tris-HCl (pH 8.8), 0.33% (w/v) ammonium persulfate. Polymerization was initiated by adding 0.044% (v/v) TEMED.[3] The stacking gel consisted of 6% acrylamide, 0.125 M Tris-HCl (pH 6.8), 1% ammonium persulfate, and 0.1% TEMED. Upper and lower gel running buffer contained 25 mM Tris, 0.2 M glycine, 0.1% SDS. The gels were run over night at 4°C at a constant current of 9 mA.

After electrophoresis, the gels were either soaked immediately in 0.2% Coomassie blue which was dissolved in 40% (v/v) methanol and 7% (v/v) acetic acid in H_2O for 3 h and destained afterwards in stain free solution as above or were prepared for autoradiography. For the latter purpose, the gels were fixed in 10% acetic acid for 1 h, soaked twice in dimethylsulfoxide for 20 min, in PPO (2,5-diphenyloxazole)-dimethylsulfoxide solution (0.2 g PPO/1 ml final volume) for 90 min, in water for 2 h with frequent changes and twice in 15% ethanol in H_2O, and placed between two layers of cellophane. The gels were air-dried and exposed to XRP 1 or XAR 5 films (Kodak).

Two-dimensional gel electrophoresis was performed as described by O'Farell (12) with the following changes: the ampholines which were added at a final concentration of 2.16% consisted of 74% ampholines (pH 5–7), 18% ampholines (pH 3.5–10), 4% ampholines (pH 2–4.5), and 4% ampholines (pH 9–11). Proteins were extracted from the plastids with phenol, precipitated from the phenol phase by adding 0.1 M ammonium acetate in ethanol, and resuspended in lysis buffer. This was done at 37°C by use of a tissue homogenizer. Samples were loaded on the acidic end of the tube gels. The gel was run, equilibrated, and loaded onto the second SDS-dimension as described by O'Farell. Electrophoresis in the second dimension was carried out as described above for one-dimensional SDS gels. The gels were silver-stained as in Morrissey (10).

Coomassie blue stain was eluted from the bands of the gel shown in Figure 1 by soaking the excised gel fragments in 40% (v/v) ethanol, 0.1% (w/v) SDS in water with frequent changes over a period of 2 d. The final volume was adjusted and absorption was read at 600 nm.

Western Blot Analysis. The polypeptides separated by gel electrophoresis in two dimensions were tranfered onto nitrocellulose (0.1 μm; pH 79; Schleicher and Schuell) at a constant current of 250 mA for 2 h. The transfer buffer consisted of 25 mM Tris, 190 mM glycine, 20% (v/v) methyl alcohol, and 0.1% (w/v) SDS (16). After two 10 min washes in TBS (150 mM NaCl, 20 mM Tris-HCl [pH 7.4]), the nitrocellulose sheet was blocked in a solution of 5% (w/v) milk protein (milk powder), 20 mM NaN_3 in TBS at 37°C for 2 h. The rabbit antibodies were then applied in 1.66% milk protein, 20 mM NaN_3 in TBS at room temperature for 18 h. After three 10 min washes in TBS, the nitrocellulose sheet was treated with 0.05 μCi ^{125}I-protein A per ml solution (TBS containing milk protein and azide as for the antibody treatment) at room temperature for 2 h. The nitrocellulose sheet was washed three times in large volumes of TBS (10 min each), dried and autoradiographed.

RESULTS

Changes in the Polypeptide Composition of Plastids during Greening. Intact plastids were isolated from 11 d old dark-grown pea plants at various times during greening and analyzed by one-dimensional SDS-gel electrophoresis. Only a few differences in the polypeptide pattern were revealed (Fig. 1). Most bands resolved in this gel are present both in etioplasts and in chloroplasts from light-grown tissue. The same amount of protein was loaded into each lane, therefore changes in individual bands indicate changes in relative abundance of the polypeptides. Several bands clearly increased in intensity during greening: the SSU (14 kD), a 22 kD protein, the LHCP (25 kD), a 39 kD protein which corresponds to a subunit of glyceraldehyde-3-phosphate dehydrogenase (as deduced from its mol wt, its abundance, and the light-inducability of its corresponding message [4, 5]) and the LSU (55 kD) are examples of this behavior. In peas, leaf development is arrested at an early stage when plants are kept in the dark (2). Upon illumination, etioplasts increase in size during their transformation into photosynthetically active chloroplasts, therefore,

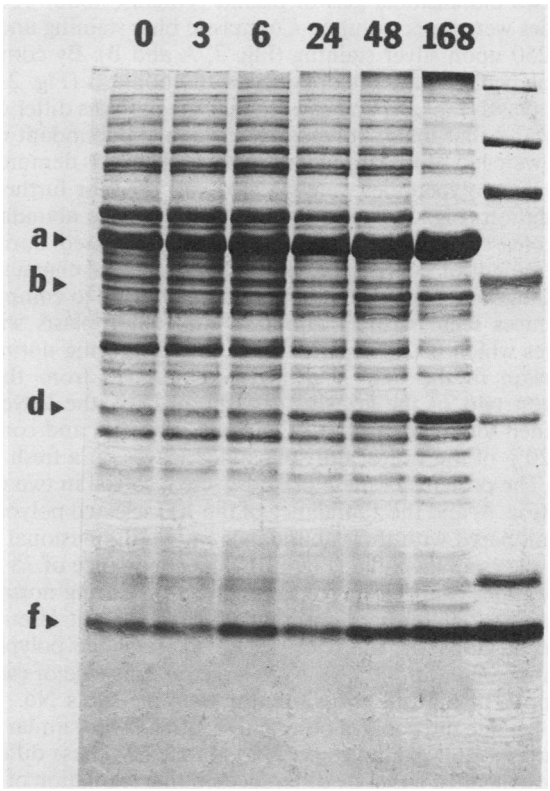


FIG. 1. Coomassie blue stained SDS-gel of polypeptides of isolated intact plastids. Plastids were isolated from plants which had been grown in complete darkness (lane 1), or under illumination for 3 h (lane 2), 6 h (lane 3), 24 h (lane 4), 48 h (lane 5), or 168 h (lane 6). Plastids were boiled in an SDS-containing solution and after centrifugation plastid proteins equivalent to 100 μg BSA in the protein assay were loaded into each lane. Lane 7 shows protein markers of the following molecular weights: 14.3, 18.4, 25.7, 43.0, 68.0, 97.4, and 200 kD. The arrows and letters refer to the LSU (a), GAPDH-subunit (b), LHCP (d), and SSU (f).

[3] Abbreviations: TEMED, *N,N,N',N'*,tetramethylethylenediamine; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; LHCP: light-harvesting Chl *a*/*b*-protein; LSU: large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; SSU: small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; PPO, 2,5-diphenyloxazole; TBS, triethanolamine-buffered saline.

an increase in polypeptide abundance in the gel must reflect a real increase on a per cell basis. A decrease in band size, as visualized for some polypeptides, for example for a 35 kD polypeptide or a polypeptide with an apparent mol wt of 54,000, may reflect a reduction in the fraction of total protein it represents and does not necessarily indicate that these proteins become less abundant on a per plastid or per cell basis. The large increase in amounts of SSU, LHCP, and LSU give the impression of a decrease in abundance of other polypeptides. This is illustrated by comparing the average protein content per organelle, *i.e.* per etioplast or chloroplast. Etioplasts contained 2 to 2.5 $\times 10^{-12}$ g protein each. Chloroplasts from 48 h greened plants had about 4.1 and chloroplasts from 10 d old plants grown continuously in a light-dark cycle had about 4.6 $\times 10^{-12}$ g protein per organelle. As a first characterization of these changes, we eluted the Coomassie stain from the bands corresponding to SSU, LHCP, and LSU and compared it with the amount of stain in all other bands. In etioplasts, less than 25% of the total Coomassie stain was found in these three bands, whereas in chloroplasts of fully greened leaves about 55% of the stain was found in the bands of SSU, LHCP, and LSU.

Both the standardization on the basis of equal protein and the resolution in one-dimensional gels seem to be inadequate to analyze changes in the whole set of plastid polypeptides. To deal with these problems, we introduced plastid number as parameter for standardization and two-dimensional gel analysis for improving the resolution.

In two-dimensional gels of plastid proteins, about 120 polypeptides were detected upon Coomassie blue staining and more than 250 upon silver staining (Fig. 2, A and B). By comparing the polypeptide patterns obtained with etioplasts (Fig. 2A) and chloroplasts (Fig. 2B) one can see that the patterns differ considerably and that many polypeptides which are abundant in etioplasts were less abundant in chloroplasts. This was demonstrated when 100 polypeptides were selected and used for further characterization (Fig. 3, A and B). Of these 100, the abundance of 39 decreased and the abundance of 46 increased during the development of etioplasts to chloroplasts. Before characterizing the changes in more detail, it seemed interesting to compare the differences seen between etioplasts and chloroplasts with the changes which occur when plastids develop during normal leaf expansion in the light. Plastids were isolated from the very youngest part of the growing pea shoot; here, the leaves were expanded to less than 8% of their final leaf area and contained only 20% of the Chl of fully expanded leaves on a fresh weight basis. The proteins of these plastids were resolved in two dimensions (Fig. 4) and the abundance of the 100 selected polypeptides was compared with their abundance in two-dimensional gels of chloroplast polypeptides (Fig. 2B). The abundance of 78 plastid polypeptides changed in the same direction during normal leaf development as during light-induced development of etioplasts (Table I). However, the pool sizes of most of the polypeptides which increased in the light were larger at the stage of early leaf development than in etioplasts, for example spots No. 17, 40, and 84, while the pools of other polypeptides were similar to that in etioplasts, for example spots No. 4 and 76. These differences can be explained either by differences in fine regulation of plastid development or by assuming that plastids in the early stage of leaf development in the light are already advanced as compared with the developmental stage of etioplasts. However, the general pattern of changes during light-induced plastid differentiation is very similar.

Identification of Thylakoid Membrane Proteins. To identify components of the thylakoid membrane in two-dimensional patterns of plastid polypeptides, a membrane fraction was prepared from chloroplasts and analyzed (Fig. 5). Stromal proteins were resolved in two dimensions as a negative control (result not shown). Twenty-four of the 100 selected polypeptides could be identified as components of the thylakoid membrane fraction by their identical positions in gels of total plastid proteins and by lack of these spots in gels of the soluble fraction. Twenty-three out of these 24 polypeptides increased in abundance significantly when etioplasts were transformed into chloroplasts (Table I). With few exceptions, all thylakoid proteins could be detected in etioplasts at least when higher protein concentrations had been loaded. Polypeptide No. 31 is an example of polypeptides which were undetectable in etioplasts. This confirms results by Grebanier *et al.* (6) who showed that only three of the large number of thylakoid membrane proteins could not be detected in maize etioplasts.

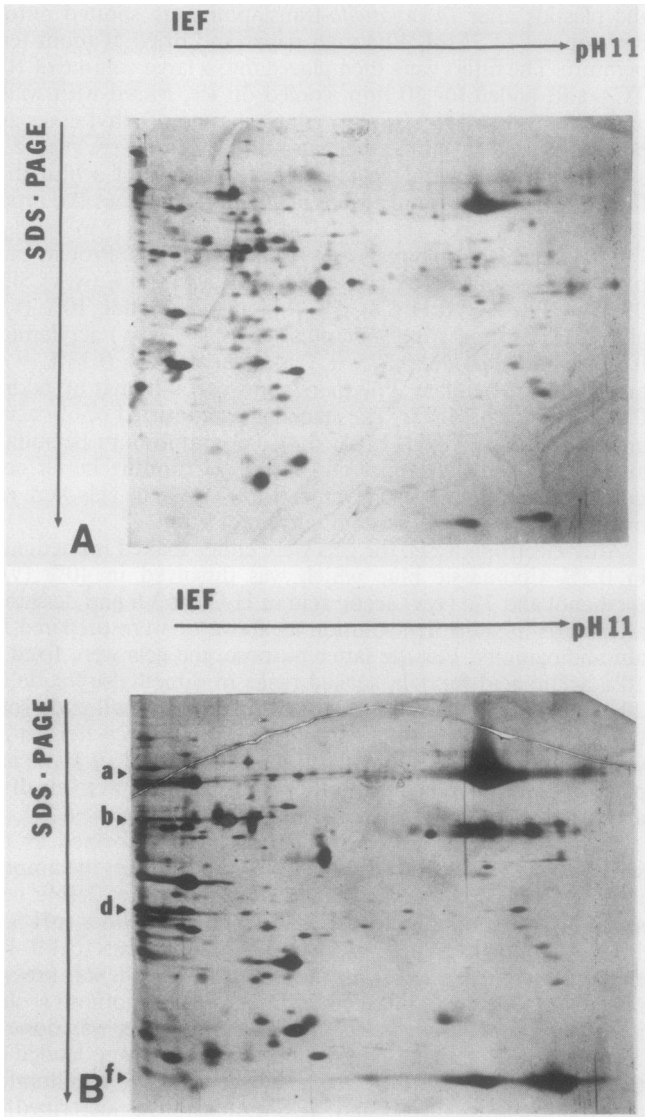
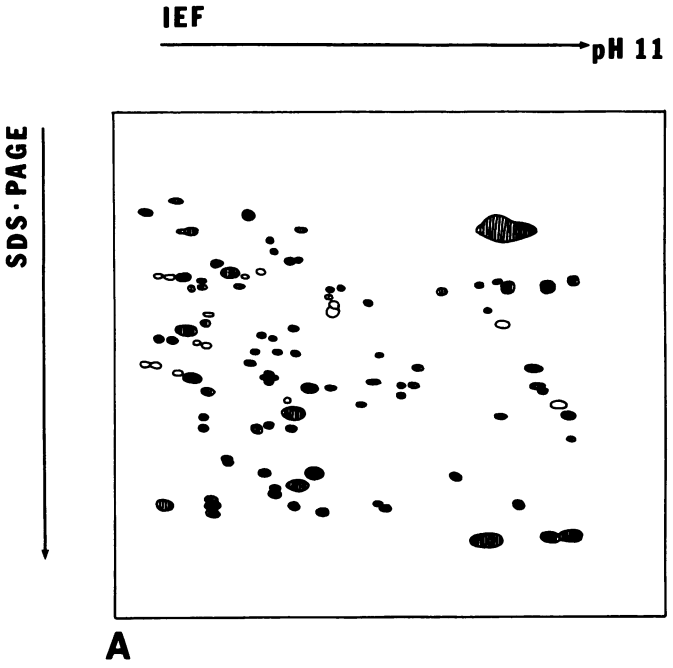


FIG. 2. Silver-stained two-dimensional gels of etioplast (A) and chloroplast polypeptides (B). Proteins of an identical number of plastids were extracted and loaded into the acidic end of a tube gel for the first dimension electrophoresis. The horizontal arrow shows the migration direction of the polypeptides; the term "pH 11" only indicates the alkaline end of the gel, not a measured pH value. The arrows and numbers in (B) refer to the positions of the LSU (a), GAPDH-subunit (b), LHCP (d), and SSU (f).

Western Blot Analysis of Plastid Proteins Resolved into Two Dimensions. In the previous section, proteins of the membrane fraction were located in gels of total chloroplast protein as a first



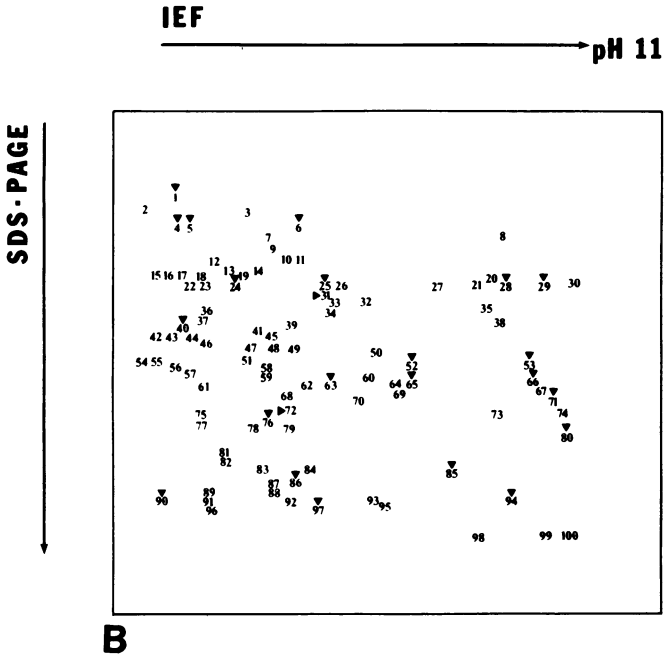


FIG. 3. A, Schematic representation of the positions on the gel after two-dimensional electrophoresis of 100 selected polypeptides as described in the text. Spots which increased in intensity during greening are shown as hatched areas, spots which decreased as filled (black) areas. The polypeptides were numbered as shown in B. The triangles mark polypeptides which were identified as membrane proteins.

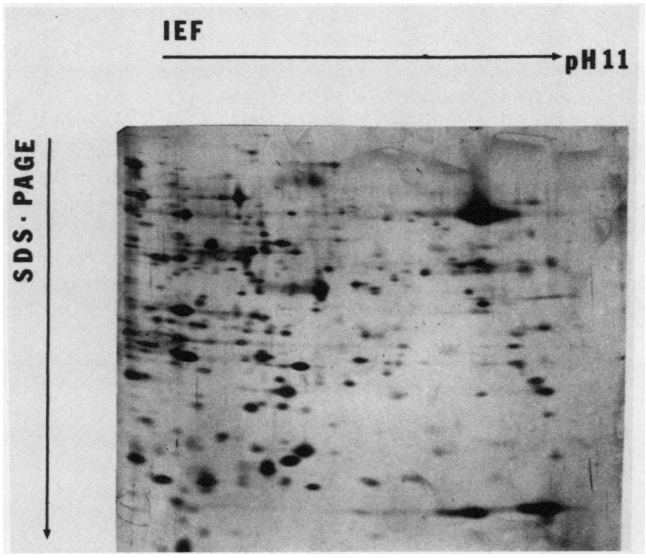


FIG. 4. Silver-stained two-dimensional gel of polypeptides of chloroplasts which had been isolated from the very youngest part of the growing shoots. See also legend to Figure 2.

step toward identifying the changes. However, a specific characterization of individual polypeptides is desirable. The complexity of polypeptide patterns in two-dimensional gels does not allow the direct identification of polypeptides from known isoelectric points and apparent mol wt. Immunological techniques can be used to specifically identify polypeptide species. Figure 6 shows a Western blot analysis of chloroplast polypeptides with antibodies which had been raised in rabbits against spinach coupling factor and ribulose-1,5-bisP carboxylase. A comparison of this fluorogram with silver stained gels of the same experiment allows the identification of the LSU, three forms of SSU, and subunits of the coupling factor I. This result shows that Western blot analysis can be used to identify polypeptides in two-dimensional gels. However, it should be pointed out that: (a) For orientation purposes standard antibodies should be included. Antiribulose-1,5-bisP carboxylase is suitable as it defines the running characteristics of the first dimension (spreading of the SSU-forms) and of the second dimension (distance between LSU and SSU). (b) Reference gels for staining should be run under identical conditions because small differences in material and loading may cause displacement of individual spots. (c) The approximate positions of the polypeptides examined in two-dimensional gels should be known, so the interpretation of the results is not endangered by background radioactivity on the nitrocellulose or by nonspecific binding of the polyclonal antibodies.

Translation by Isolated Intact Plastids. In plastid development, the production of plastid proteins by the nuclear-cytoplasmic system must be coordinated with the availability of proteins of plastid origin. Mullet *et al.* (11) improved the *in organello* translation system of isolated chloroplasts by using pulse-chase labeling; the chase period allows completion of the translation to full length products, thereby reducing the number of apparent products. In order to investigate differences in *in organello* translation by isolated plastids during photoregulated transformation of etioplasts to chloroplasts and to further characterize the changes observed in the polypeptide patterns during greening, plastids isolated from dark-grown plants or from plants illuminated for 3, 6, 24, 48, or 168 h were used for *in organello* translation (Fig. 7). Three different classes of response to illumination could be distinguished: (a) The 32 kD polypeptide was not detectable among the translation products of etioplasts (*cf.* [15]) but became, after LSU, the second most dominant product in chloroplasts. (b) Synthesis of several polypeptides decreased; for example, a 17 and a 21 kD polypeptide. (c) Synthesis of most polypeptides did not change significantly. In chloroplasts from completely light-grown pea plants, the high rates of synthesis of LSU and 32 kD-second electron accepting plastoquinone of PSII-protein give the impression that fewer proteins are synthesized than at earlier stages of plastid development when equivalent amounts of labeled polypeptides are compared. Longer exposure of the gels reveals that there are no pronounced qualitative differences between the *in organello* translation products of

Table I. *Partial Characterization of 100 Plastid Proteins*

One hundred of the polypeptides detected in silver-stained gels of plastid proteins were selected for further characterization (Fig. 3). Their apparent mol wt and their relative positions in polyacrylamide gel isoelectric focusing analyses are given in the second and third columns, respectively. The value given for the position in isoelectric focusing (1.D) was calculated by dividing the distance of the spot from the acidic end of the gel by the total length of the gel. A value of 10 corresponds to a pH of approximately 5, a value of 80 to pH 7. The differences in polypeptide abundance between etioplasts and chloroplasts from 10 d old leaves (E-C: *cf.* Fig. 2, A and B) and between chloroplasts isolated from very young leaves and chloroplasts from 10 d old leaves (C-C: *cf.* Fig. 4 and Fig. 2B) are given in the fourth and fifth columns: + stands for increasing, − for decreasing abundance, 0 for no change, and mixed symbols (0−, 0+, and −0) define intermediate trends. Some of the polypeptides were identified as *in organello* translation products (*in org* column; *cf.* Fig. 8) or as polypeptides being imported from poly(A$^+$) mRNA translation products in a posttranslational uptake system (post-trl column; *cf.* Figs. 4 and 5 in (5); the numbers in parentheses refer to the numbers used in this communication). The symbols (as described above) indicate differences between dark-grown plants and completely light-grown plants. Some of the polypeptides were further characterized as components of the membrane fraction of chloroplasts or as specific polypeptides (other column).

No.	MW_r	1.D	E-C	C-C	*in org.*	post-trl.	Other
1	95	22	+	+			Thylakoid
2	72	16.5	−	−			
3	72	34.6	−	−		− (6)	
4	56	23.1	+	+	+		Thylakoid
5	56	25	+	+	+		Thylakoid
6	56	45	+	+			Thylakoid
7	55	38.8	−	−			
8	55	80.8	+	+	+		LSU
9	45	39.2	−	−			
10	42	40.7	−	−		0− (21)	
11	42	43.1	−	−		0− (22)	
12	41	30	−	−		− (19)	
13	39	31.5	+	+		+ (31)	GAPDH
14	39	37.7	0	0		+ (32)	
15	37	18.8	0+	0			
16	37	20.8	0+	0			
17	37	23.5	+	+			
18	36	26.9	+	+			
19	36	34.6	0	−			
20	36	81.1	−	−			
21	35	76.9	+	0			
22	35	24.6	+	+			
23	35	26.9	+	+			
24	35	33.8	+	+		+ (42)	Thylakoid
25	34	50	+	+		0+ (36)	Thylakoid
26	35	52.3	−	−			
27	34	70.8	+	+			
28	34	83.1	+	+	+		Thylakoid
29	34	90	+	+	+		Thylakoid
30	36	94.6	+	+			
31	33	50	+	+		+ (47)	Thylakoid
32	32	57.3	−	−			
33	32	50.8	0	0			
34	30	50.4	0	0			
35	30	79.2	−	−			
36	30	28.1	0	0			
37	29	26.9	+	−		+ (59)	
38	29	81.5	0	0−			
39	28	43.1	−	−			
40	28	23.8	+	+		0 (64)	Thylakoid
41	28	37.7	−	−			
42	27	18.8	−	−			
43	27	21.5	+	+			
44	27	26.2	0	0−			
45	27	40	−	−		+ (65)	
46	27	28.1	0	0−			
47	26	36.2	−	−			
48	26	40.8	−	−		− (75)	
49	25	43.8	−	−		− (76)	
50	25	59.2	−	−			
51	25	35.4	−	−			
52	25	66.9	+	0			Thylakoid
53	24	88.1	+	+			Thylakoid
54	25	16.5	0+	0+			
55	25	18.1	0+	0+		0 (90)	
56	24	22.7	0	0−			
57	24	25.4	−	−		− (94)	
58	24	39.2	−	−			
59	24	39.2	−	−			
60	23	58.5	+	0−			
61	23	28.5	+	0−			
62	23	43.1	−	−		− (98)	
63	23	46.5	+	+			Thylakoid
64	23	63.8	−	−			
65	23	66.2	+	0			Thylakoid
66	23	38.3	+	0−			Thylakoid
67	23	89.2	−	−			
68	22	43.1	0	0−			
69	23	55.8	−	−			
70	22	56.2	−	−		− (102)	
71	22	92.3	0	0−		0 (101)	Thylakoid
72	22	43.8	+	+			Thylakoid
73	22	81.5	−	−			
74	22	93.9	−	−		− (105)	
75	21	27.3	+	0			
76	21	35.4	+	+		+ (106)	−SU-CFI/Thylakoid
77	21	26.9	+	0		−0 (112)	
78	21	33.1	+	−			
79	21	43.1	−	−		− (108)	
80	20	94.6	+	+			Thylakoid
81	20	31.5	−	−		− (120)	
82	19	31.2	−	−			
83	19	38.5	+	0+			
84	19	47.7	−0	−0		0 (122)	
85	19	73.8	+	+	+		Thylakoid
86	19	44.6	+	+			Thylakoid
87	19	40.8	−	−			
88	18	40.4	−	−			
89	18	28.5	+	0			
90	18	20.4	+	0			Thylakoid
91	18	28.5	+	0		0 (127)	
92	18	43.8	−0	−			
93	18	59.2	−0	−			
94	18	84.6	+	0+	+		-SU-CFI/Thylakoid
95	18	59.2	−	−			
96	17	28.5	+	+			
97	17	49.2	+	+			Thylakoid
98	14	80.8	+	+		+ (131)	SSU
99	14	90.8	+	+		+ (132)	SSU
100	14	94.6	+	+		+ (133)	SSU

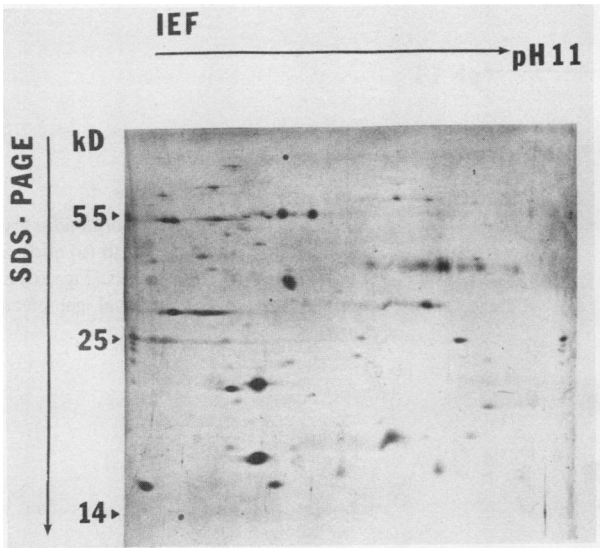


FIG. 5. Silver stained two-dimensional gel of thylakoid membrane proteins. Thylakoids were obtained from chloroplasts which were isolated from fully expanded, 10 d old pea leaves and were extensively washed in 5 mM sodium phosphate buffer (pH 7.2) prior to protein extraction with phenol as described above.

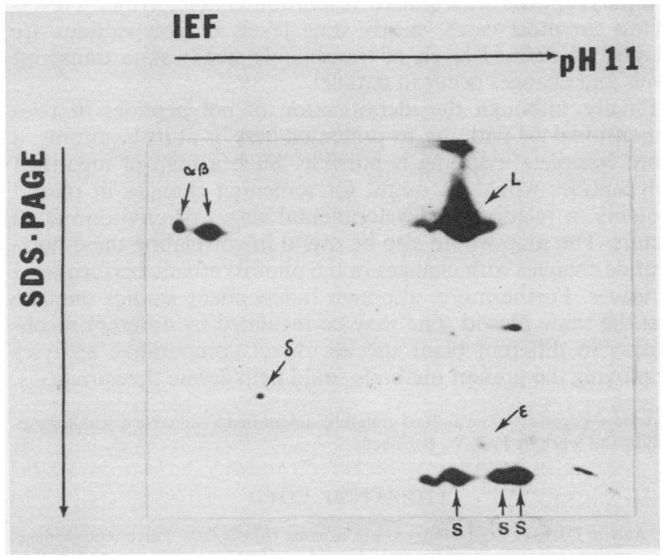


FIG. 6. Autoradiography of a western blot of a two-dimensional gel of chloroplast polypeptides as shown in Figure 2b. Antibodies against spinach ribulose 1,5-bisP carboxylase and spinach coupling factor CFI were used in this experiment.

chloroplasts from dark-grown 48 h greened and light-grown pea plants. On a protein basis, the chloroplasts incorporated about three times as much [^{35}S]methionine into translation products as etioplasts.

The *in organello* translation products obtained with etioplasts and chloroplasts were resolved in two dimensions. The results are shown in Figure 8. About 80 translation products can be seen. Major qualitative differences cannot be seen in the general pattern of translation products synthesized by etioplasts and chloroplasts. When the gels of the *in organello* translation products of etioplasts were exposed for longer, almost all polypeptides synthesized in chloroplasts and resolved by the two-dimensional gel could be detected. However, at least 15 polypeptides show largely enhanced synthesis in chloroplasts as compared to etioplasts. Only a small number of translation products, *i.e.* 14, can

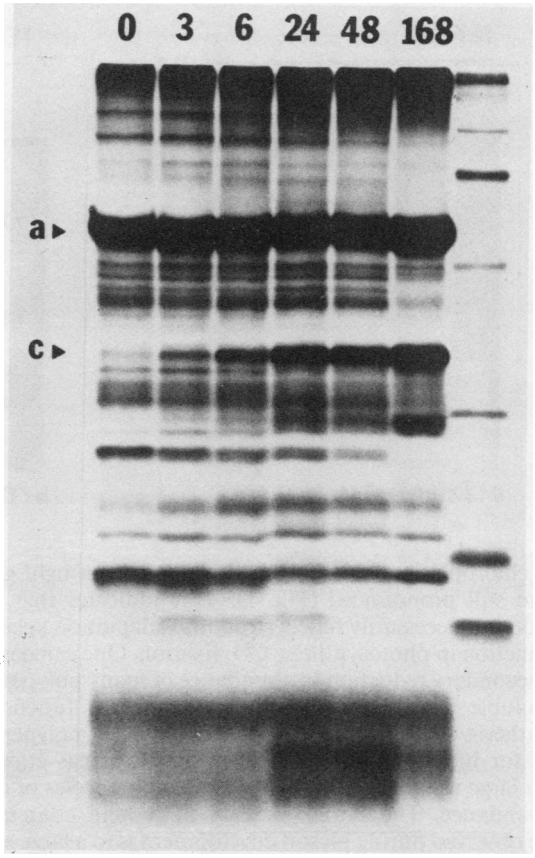


FIG. 7. *In organello* translation by plastids isolated from completely dark grown (lane 1), 3 h (lane 2), 6 h (lane 3), 24 h (lane 4), 48 h (lane 5), or 168 h (lane 6) light grown pea plants. Lane 7 shows the mol wt standards of 14.3, 18.4, 25.7, 43.0, 68,0, 97.4, and 200 kD. The arrows and letters on the left side of the gel refer to the position of LSU (a) and of the 32 kD-polypeptide (c).

be identified in silver-stained gels of plastid proteins, 7 of which correspond to polypeptides of the group of 100 selected polypeptides (Table I). Possible explanations for the low number of polypeptides which could be identified in silver-stained gels are: (a) Some of the polypeptides are not full length products even when the period of incorporation of [^{35}S]methionine is followed by a chase period. (b) Secondary modifications of the synthesized polypeptides cannot occur in the chloroplasts. (c) Some of the products of *in organello* translation do not accumulate *in vivo* to amounts which could be detected by silver staining.

DISCUSSION

Although there is a large amount of published data dealing with changes in plastid composition during greening (8, 17), comparisons of changes in polypeptide composition with those during regular leaf development in the light are missing. Analysis of plastid polypeptide composition by one- or two-dimensional gel electrophoresis reveals that the number of polypeptides present in pea chloroplasts and absent in etioplasts is small. Although the number of dark and light specific polypeptides is small, the changes in relative abundance of individual polypeptides are pronounced. At least 28 of the 46 polypeptides which increased in abundance per organelle function in photosynthesis; they could be identified as subunits of the ribulose-1,5-bisP carboxylase or the GAPDH or could be localized in the thylakoid membrane. The light-dependent differentiation of pea etioplasts into chloroplasts is not completed within 48 h; the differences

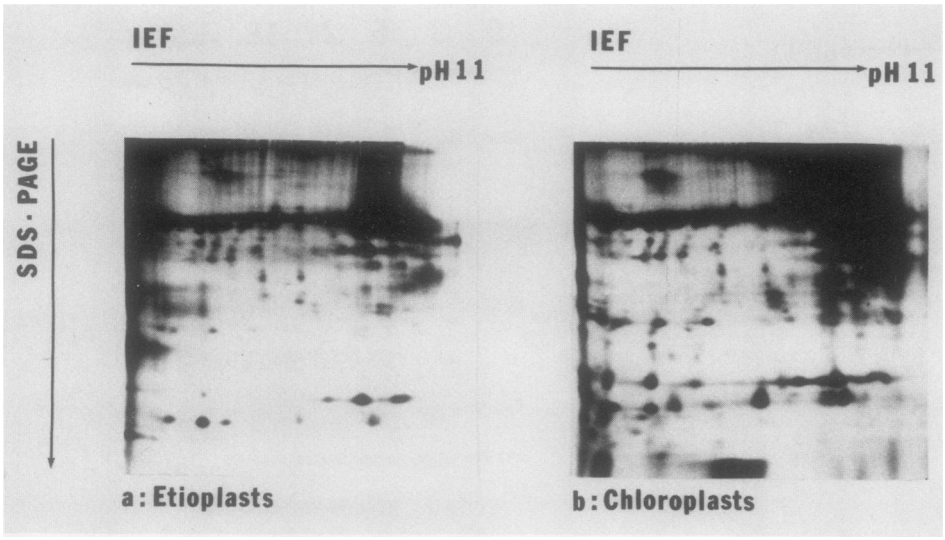


FIG. 8. Fluorogram of *in organello* translation products obtained with (a) etioplasts (Fig. 7a) and (b) chloroplasts (Fig. 7b) and resolved by two-dimensional gel electrophoresis.

between chloroplasts of 48 h greened plants and of light grown plants are still pronounced (Fig. 1). This indicates that these changes do not necessarily reflect structural adaptation necessary for the function in photosynthetic CO_2 fixation. One can describe this as a secondary reduction in abundance of many polypeptides of the soluble fraction which probably have no function in photosynthesis. In chloroplasts, a small number of polypeptides accounts for most of the organelle protein, whereas etioplasts contain a large number of different polypeptide species of about equal abundance. The 2-fold increase in protein content per organelle observed during plastid development is to a large extent due to an accumulation of a few polypeptides, such as the subunits of the ribulose-1,5-bisP carboxylase/oxygenase, GAPDH and LHCP.

Although the excellent resolution of polypeptides in two-dimensional gels makes this technique suitable for monitoring the changes in the whole set of abundant plastid proteins, its limitation lies in the difficulty of identifying specific polypeptides. The beginning of such an analysis is presented in this communication and summarized for 100 selected polypeptides in Table I. The transcriptional-translational origin of 36 of the 100 polypeptides can be traced back by comparing stained two-dimensional gels of plastid polypeptides with autoradiographed gels of *in organello* translation products and with autoradiographed gels of polypeptides which had been synthesized from poly(A^+) mRNA preparations in a cell-free system and which had been posttranslationally imported by chloroplasts *in vitro* (for the detailed description see [5]). this relatively low percentage of polypeptides which can be identified needs some explanation (18): (a) Only methionine-containing polypeptides can be detected. (b) The cell-free translation system may differ from the *in vivo* translation apparatus. (c) The message levels for some polypeptides with low metabolic turnover may be too low to be seen in fluorograms of posttranslationally imported polypeptides.

For a first analysis, the 36 polypeptides were taken as a basis for comparing the changes in polypeptide level with the changes in transcript levels. (The production of labeled polypeptide by *in organello* translation is considered to give at least a relative estimate of changes in the transcript level. This is not necessarily true, as translational control is known to occur for some plastid-encoded polypeptides in other species [3, 9].) In the seven cases where the polypeptide was synthesized on plastid ribosomes, the observed increase in abundance of the protein was matched by an increase in transcript level during greening. Changes in abundance of 20 of the 29 polypeptides which were synthesized on cytoplasmic ribosomes were accompanied by parallel changes in translatable transcripts. In seven cases either the transcript level or the level of protein stayed constant while the other parameter changed. In only two cases, changes showed opposite trends; however, in these two cases (spots No. 45 and 77) the observed bands in the silver-stained gel might in fact be double bands of two polypeptides with similar resolution characteristics. Nevertheless, in most cases, steady state levels of polypeptides are coupled to defined levels of translatable steady state transcript levels and changes occur in parallel.

Finally, although the identification of polypeptides in two-dimensional gel patterns, as presented here, is at its beginning, a more complete mapping is possible. Such a map of identified polypeptides would be useful for screening changes in plastid proteins in relation to developmental stages or environmental factors. The map would also be useful in correlating these polypeptide changes with changes in the photosynthetic performance of leaves. Furthermore, although independent studies indicate that the same plastid gene may be regulated by different mechanisms in different plant species, direct comparative analyses employing the present methods could help define the variations.

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